Article

Molecular and morphological characterization of *Amathia distans* Busk and *Amathia brasiliensis* Busk (Bryozoa: Ctenostomata) from the tropical and subtropical Western Atlantic

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Abstract

Morphological and molecular analyses have proven to be complementary tools of taxonomic information for the redescription of the ctenostome bryozoans *Amathia brasiliensis* Busk, 1886 and *Amathia distans* Busk, 1886. The two species, originally described from material collected by the 'Challenger' expedition but synonymized by later authors, now have their status fixed by means of the selection of lectotypes, morphological observations and analyses of DNA sequences described here. The morphological characters allowing the identification of living and/or preserved specimens are (1) *A. brasiliensis*: whitish-pale pigment spots in the frontal surface of stolons and zooids, and a wide stolon with biserial zooid clusters growing in clockwise and anti-clockwise spirals along it, the spirality direction being maintained from maternal to daughter stolons; and (2) *A. distans*: bright yellow pigment spots in stolonal and zooidal surfaces including lophophores, and a slender stolon, thickly cuticularized, with biserial zooid clusters growing in clockwise and anti-clockwise spirals along it and the spirality direction not maintained from maternal to daughter stolons. Pairwise comparisons of DNA sequences of the mitochondrial genes cytochrome c oxidase subunit I and large ribosomal RNA subunit revealed deep genetic divergence between *A. brasiliensis* and *A. distans*. Finally, analyses of those sequences within a Bayesian phylogenetic context recovered their genealogical species status.

Key words: redescription, genealogical species, COI, 16S rRNA, Vesiculariidae

Introduction

Amathia Lamouroux, 1812 is represented by approximately 33 extant described species of Bryozoa, distributed across all oceans (Bock 2010), and occurring in fouling, intertidal and subtidal habitats. While many of the species seem to be restricted to specific regions, at least eight are currently considered to be widespread (Bock 2010). Recent research relying on DNA sequence data has challenged the natural cosmopolitan status attributed to other bryozoan species (e.g. Davidson & Haygood 1999; Schwaninger 1999, 2008; Hoare *et al.* 2001; McGovern & Hellberg 2003; Mackie *et al.* 2006; Nikulina *et al.* 2007; Nikulina 2008; Hughes *et al.* 2008), classifying them instead as complexes of cryptic species, and/or recognizing events of bioinvasion responsible for their present widespread patterns of distribution. Specifically for *Amathia*, misidentifications, incomplete descriptions, and wrong taxonomic decisions leading to erroneous synonymies have underlined many previous references concerning several of its species, as reported by Chimonides (1987). This situation has not changed, as noted more than 20 years later by Souto *et al.* (2010). Additionally, there are still no DNA sequences available that might help to clarify their taxonomic status (only two sequences of ~70 base pairs of GRT-G1 retrotransposon gypsy-like reverse transcriptase, and the pseudogene LRT-L1 retrotransposon LINE-like reverse transcriptase, have been deposited in GenBank for *Amathia convoluta* Lamarck, 1816).

Recently, Vieira *et al.* (2008) listed four species of the genus as occurring in Brazilian waters: *Amathia brasiliensis* Busk, 1886, *Amathia crispa* Lamarck, 1816 (=*Amathia convoluta* Lamouroux, 1816, see d'Hondt 1983),

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Amathia distans Busk, 1886, and *Amathia vidovici* Heller, 1867. Busk (1886) described *A. brasiliensis* and *A. distans* from Northeastern Brazil (Bahia State), and distinguished the two species by differences in zooidal form and size, stolon diameter, and the way zooid clusters were distributed along the stolons. Specimens attributed to *A. brasiliensis* and *A. distans* have been reported at various localities for more than a century (MacGillivray 1895; Harmer 1915; Hastings 1927; Marcus 1937, 1941, 1949, 1955; Osburn 1940; Winston 1982; d'Hondt 1983; Chimonides 1987; Souto *et al.* 2010). Most of those taxonomic studies were part of faunal surveys rather than phylogenetic revisions, but there were attempts to synonymize the two species in the years since their original description. Harmer (1915) upheld Busk's species, as did Osburn (1940), but Marcus (1937: p. 134) combined the two as *A. distans,* using the name and description appearing first in Busk's report, and his decisions seem to have been generally accepted by later authors.

The objective of this work was to test the taxonomic validity of *A. brasiliensis* and *A. distans*, based on morphological redescription, molecular characterization and phylogenetic analyses of nucleotide sequence data. The main purpose of doing phylogenetic search was to test the validity of *A. brasiliensis* and *A. distans* as genealogical species (i.e., whether they are reciprocally monophyletic; Baum & Shaw 1995; Hudson & Coyne 2002). DNA sequences were obtained for the first time for the four species occurring on Brazilian shores. The revised taxonomic status of these two species is expected to form the basis for future studies, which may examine the cosmopolitan status of putative conspecifics from other regions of the world.

Material and methods

Sampling. *Amathia brasiliensis* (n=10), *A. distans* (n=06), *Amathia* cf. *crispa* (n=02), and *Amathia* cf. *vidovici* (n=04) were collected in the intertidal or shallow sublittoral from localities along the northeast, southeast and south of the Brazilian coast (Table 1). The latter two species were included only for comparative purposes and do not represent attempts to readdress their taxonomic validity – this question is being assessed in an ongoing, more inclusive study by the authors.

TABLE 1. List of taxa, site of sampling, museum and GenBank accession numbers of specimens included in present study. Legends: AL, Alagoas State; SC, Santa Catarina State; SP, São Paulo State; PR, Paraná Sate, BR, Brazil. *, data previously published; **, material from CEBIMar-USP collection.

Amathia species	Locality (site, city, state, country)	Geographic coordinates	Collection numbers	COI GenBank	16S GenBank
A. brasiliensis					
	Submarine Outfall, Maceió, AL, BR	09º40'53.69"S; 35º43'28.31"W	MZUSP 495	JF490055	JF490051
	Pajuçara, Maceió, AL, BR	09º40'05.59"S; 35º42'50.64"W	MZUSP 505	JF490055	JF490051
	Farming, S. Francisco do Sul, SC, BR	26º14'37.46"S; 48º38'17.72"W	MZUSP 497	JF490056	
			MZUSP 507	JF490056	JF490051
			MZUSP 496	JF490055	
	Canal, S. Sebastião, SP, BR	23º49'60.00"S; 45º22'60.00"W	MZUSP 506	JF490055	JF490051
	Petrobrás, S. Sebastião, SP, BR	23º41'43.95"S; 45º24'1.22"W	MZUSP 492	JF490056	JF490051
	Araçá, S. Sebastião, SP, BR	23º48'37.13"S; 45º26'27.66"W	MZUSP 493	JF490055	JF490051
			MZUSP 504	JF490055	JF490051
	Private pier, Ilhabela, SP, BR	23º46'34.14"S; 45º21'30.53"W	MZUSP 494	JF490055	JF490051

TABLE 1. (continued)

Amathia species	Locality (site, city, state, country)	Geographic coordinates	Collection numbers	COI GenBank	16S GenBank
A. distans					
	Praia Preta, S. Sebastião, SP, BR	23º49'15.02"S; 45º24'38.25"W	MZUSP 498	JF490058	
			MZUSP 500		JF490053
	Praia Grande, S. Sebastião, SP, BR	23º49'27.15"S; 45º24'48.17"W	MZUSP 499	JF490058	
			MZUSP 508	JF490058	JF490053
			MZUSP 509	JF490058	JF490053
	Canal, S. Sebastião, SP, BR		MZUSP 510	JF490058	JF490053
Amathia cf. *crispa*					
	M. Sabão, Ilha do Mel, PR, BR	25º33'57.21"S; 48º17'54.44"W	A15**	JF490057	JF490052
			A16**	JF490057	JF490052
Amathia cf. *vidovici*					
	Encantadas, Ilha do Mel, PR, BR	25º34'26.38"S; 48º19'6.66"W	A11**	JF490059	JF490054
	M. Sabão, Ilha do Mel, PR, BR		A07**	JF490059	JF490054
	Pontal da Cruz, S. Sebastião, SP, BR	23º46'50.59"S; 45º23'50.16"W	A13**	JF490059	JF490054
			A21**	JF490059	JF490054
Outgroup species	Locality (site, city, state, country)	Geographic coordinates	Collection numbers	COI GenBank	16S GenBank
Zoobotryon verticillatum					
	Araçá, S. Sebastião, SP, BR		Z1**	JF490060	JF490050
			Z2**	JF490060	JF490050
	Canal, S. Sebastião, SP, BR		Le1**	JF490060	JF490050
*Flustrellidra hispida**					
	Mumbles, Wales, UK	51°34'21"N; 03°59'28"W		NC008192	NC008192
	Aberystwyth, Wales, UK	52°25N; 04°05'W			

Morphological examination. Living colonies were examined and measured, and specimens from the municipality of São Sebastião (São Paulo State) were photographed using a digital camera mounted on a Zeiss SV 11 Stereo Microscope. The tissues were then preserved in 92–99% ETOH, and stored at -20°C until the genomic DNA (gDNA) extractions were performed.

All colonies were deposited as vouchers at Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil (MZUSP) and at The Natural History Museum, London, U.K. (NHMUK). Table 1 indicates their geographical provenance, GenBank accession and museum numbers. Comparative materials included type specimens deposited at NHMUK. The redescriptions followed the criteria proposed by Chimonides (1987) and recently used by Souto *et al.* (2010). The presence and color of pigment spots were observed in living colonies.

DNA analyses. Total gDNA was extracted with the DNeasy Blood & Tissue Kit (QIAGEN). Approximately 20 to 50 clean zooids per colony, clearly containing polypides, were used for each extraction. The primer sets LCO1490 + HCO2198 (Folmer *et al*. 1994) and SHA + SHB (Cunningham & Buss 1993) were used for PCR and sequencing of the mitochondrial genes cytochrome c oxidase subunit I (COI), and large ribosomal RNA subunit (16S rRNA), respectively. PCRs were carried out in 25µl volumes (1µl of gDNA template at [~30ng/µl], 0.8µl of each primer at [10µM], 22.4µl of H_2O), using illustra PuReTaq Ready-To-Go™ PCR Beads (GE Healthcare). Cycling conditions for the amplification were: 94º C, 5' – 35x: 94º C, 30"; 50/54º C, 30"; 72º C, 45" – 72º C, 3'.

PCR products were cleaned with illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Sequencing into both directions was performed on an ABI PRISM® 3100 Genetic Analyzer, using BigDye®Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Complementary strands were combined and edited with CodonCode Aligner (CodonCode Corporation) and compiled using Geneious Pro v5.3.6 (Drummond *et al.* 2010). The resulting sequences, checked using BLAST tool (www.ncbi.nih.gov/BLAST/), were deposited in GenBank (Table 1).

Sequences were aligned in Geneious Pro using the algorithm of the option 'Geneious alignment', under default parameters of the cost matrix 'Identity (1.0/0/0)'. Pairwise analyses were conducted in DnaSP v5 (Librado & Rozas 2009) to obtain the number of haplotypes and synonymous and nonsynonymous substitutions, nucleotide diversity 'Pi' (the average number of nucleotide differences per site between two sequences), and percentage values of variable and invariable sites.

Phylogenetic analysis. The ctenostomes *Zoobotryon verticillatum* delle Chiaje, 1828 (Vesiculariidae) and *Flustrellidra hispida* (Fabricius, 1780) (Flustrellidridae) were used as outgroup taxa. The concatenated dataset was analyzed using Bayesian inference (BI) with MrBayes v3.1.2 (Ronquist & Huelsenbeck 2003). The COI and 16SrRNA partitions were performed under the GTR+I+G model, separately estimated by Modeltest v3.7 (Posada & Crandall 1998) through Akaike Information criterion (AIC). The codon nucleotide model (Goldman & Yang 1994), using the invertebrate metazoan code available in MrBayes, was also applied for the COI partition. The analysis was run for 4,500 generations, until the standard deviation of split frequencies had reached values below 0.01. Chains were sampled every 10th generation, and 'burnin' was set to 112. The analysis was run twice, in order to verify convergence between runs. Nodal support was expressed as posterior probabilities (pp), with pp < 0.95 judged as not significant.

Systematic account

Phylum Bryozoa Ehrenberg, 1831

Class Gymnolaemata Allman, 1856

Order Ctenostomata Busk, 1852

Suborder Euctenostomata Jebram, 1973

Superfamily Vesicularioidea Johnston, 1847

Family Vesiculariidae Hincks, 1880

Genus *Amathia* Lamouroux, 1812

***Amathia brasiliensis* Busk, 1886**
(Figs 1, 2, 5, 7, 9; Tables 1–2)

Amathia brasiliensis Busk, 1886: 34, pl. 7, fig. 2; Osburn 1940: 339.
Amathia distans: Marcus 1937: 134 (part), pl. 27, fig. 72; 1955: 313 (part); Maturo 1957: 23, fig. 12; d'Hondt 1983: p. 65 (part). Non Busk 1886: 33.

Material examined. *Lectotype* (chosen here): NHMUK 1887.12.9.928 (slide mounted by A.B. Hastings using Borax-carmine Glycerine; Figures 1 and 2), *Amathia brasiliensis* (G. Busk det.), H.M.S. 'Challenger', Bahia (Brazil), 10–20 fms (18.29–36.58 m). *Paralectotypes*: NHMUK 1887.12.9.927, *Amathia brasiliensis* (G. Busk det.), H.M.S. 'Challenger', Bahia (Brazil), 10–20 fms (18.29–36.58 m); NHMUK 1890.4.14.5, *Amathia brasiliensis*, Bahia (Brazil), G. Busk Supplementary Collection; NHMUK 1899.7.1.4520, *Amathia brasiliensis*, H.M.S. 'Challenger', Bahia (Brazil), G. Busk Collection; NHMUK 1963.2.12.356, *Amathia brasiliensis*, H.M.S. 'Challenger', Bahia (Brazil), Dundee Collection. Additional material (Table 1): MZUSP 495, MZUSP 505 (donated to

NHMUK), Maceió, Alagoas State, Brazil; MZUSP (492, 493), MZUSP (504, 506) (donated to NHMUK), São Sebastião, São Paulo State, Brazil; MZUSP 494, Ilhabela, São Paulo; MZUSP (496, 497), MZUSP 507 (donated to NHMUK), São Francisco do Sul, Santa Catarina State, Brazil.

Redescription. Colonies cuticularized, erect, densely branched, brownish. Frontal surface of stolons and zooids delicate, with inconspicuous whitish-pale pigment spots observed only in living colonies; colonies in alcohol whitish-brown in color. Autozooids in biserial clusters, 8 to 18 pairs in long complete clockwise or anticlockwise spirals along a wide stolon; the direction of spirals maintained from maternal to daughter stolons. Clumps occupying from half to three quarters of distal portion of stolon. Autozooids tubular, most of them almost completely unjoined, rarely joined at one third of their total length, not varying in size. Orifice terminal, approximately subquadrangular. Rhizoids sometimes present, arising in the proximal end of stolon. Polypides with 8 equal straight tentacles, measuring approximately 0.27 mm in length.
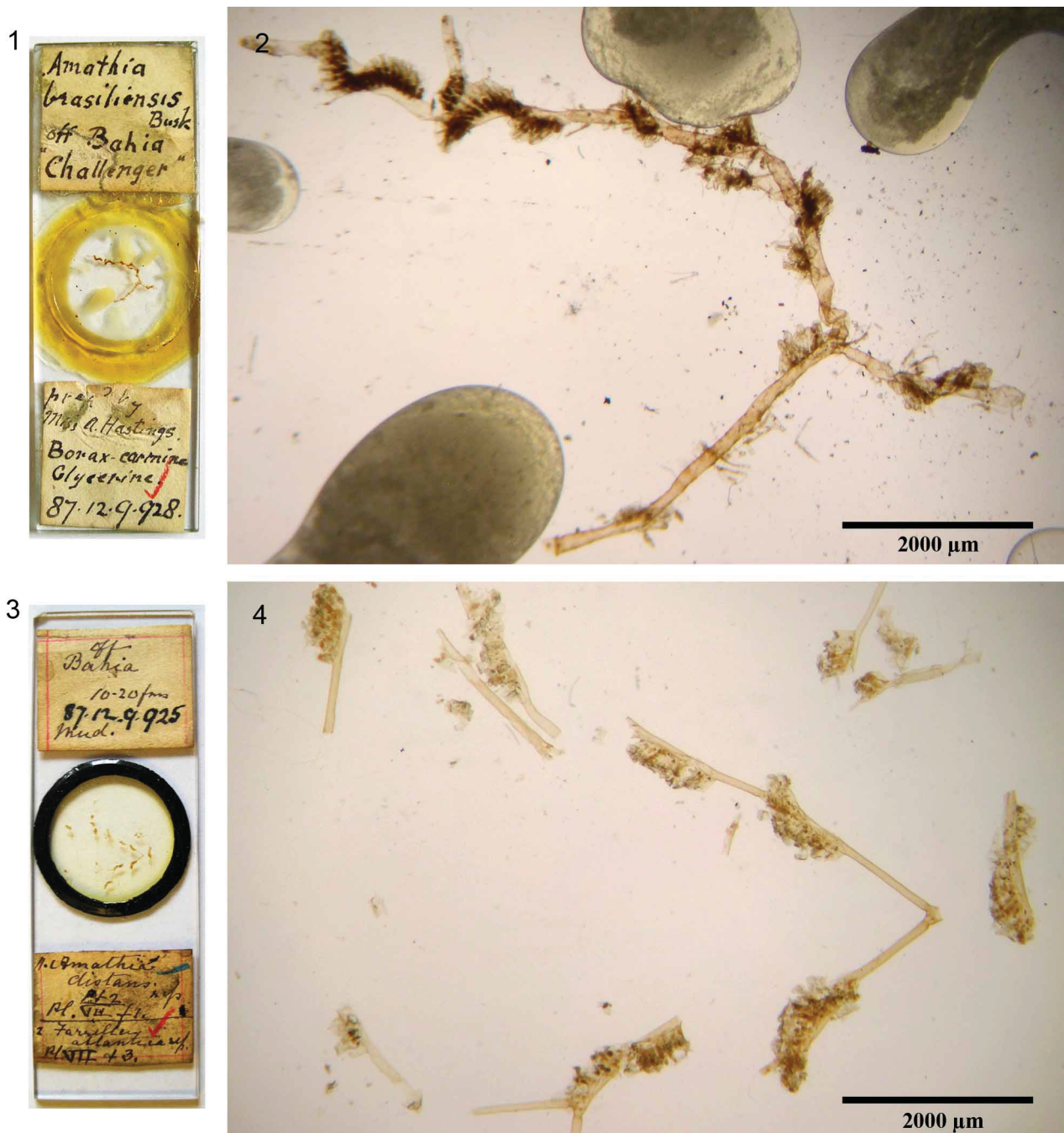
FIGURES 1–4. 1–2, *Amathia brasiliensis*, NHMUK 1887.12.9.928 (lectotype specimen, chosen here), off Bahia, Brazil; **3–4**, *Amathia distans*, NHMUK 1887.12.9.925 (lectotype specimen, chosen here), off Bahia, Brazil.
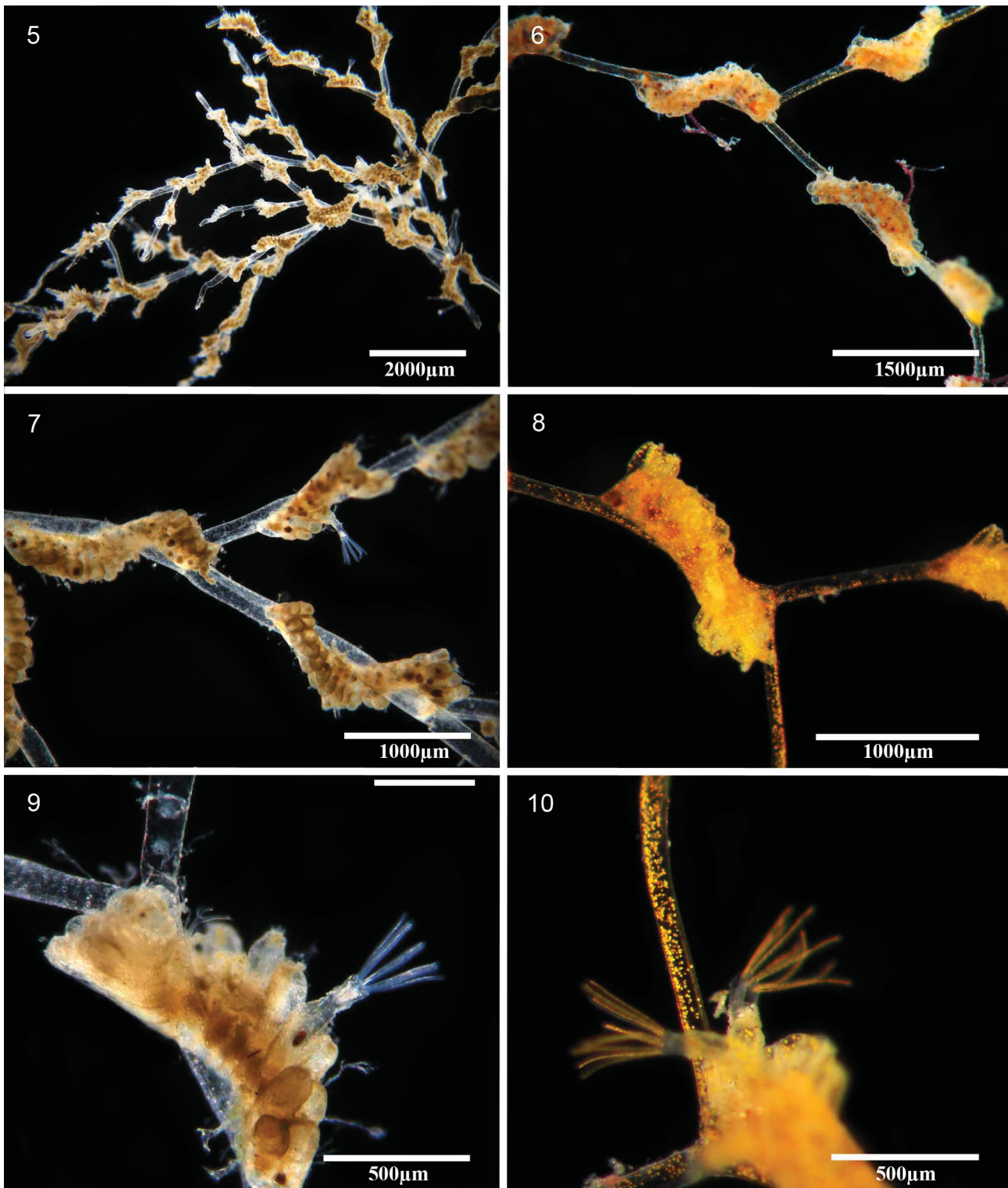
FIGURES 5–10. 5, *Amathia brasiliensis,* general view of the whitish-pale living colony; **6,** *Amathia distans,* general view of the bright yellow living colony; **7,** *A. brasiliensis,* close-up of the biserial autozooidal clusters around the wide stolon; **8,** *A. distans,* close-up of autozooidal clusters around the slender stolon; **9,** *A. brasiliensis,* close-up of whitish-pale pigment spots in the frontal surface of stolon and zooids; **10,** *A. distans,* close-up of bright yellow pigment spots in stolon and zooidal surfaces, including lophophores.

Remarks. *Amathia brasiliensis* was originally described from Bahia State, Brazil (Busk 1886). This species is characterized by whitish-pale pigment spots in the frontal surface of stolons and zooids, and by a wide stolon (about 0.22 mm of diameter) with biserial zooid clusters growing in clockwise or anticlockwise spirals along it.

Busk (1886) noted a tendency of direction of spirals, characteristic of this species. Osburn (1940) disagreed with the synonymization of the name *A. brasiliensis* under *A. distans* adopted by Marcus (1937), and used the epithet *brasiliensis* for specimens with wider stolons (0.18–0.30 mm). The North Carolina specimens reported by Maturo (1957) under the name *A. distans* have a wider stolon than *A. distans* from Brazil (see Tables 2–3 for comparison), and hence probably belong to *A. brasiliensis*.

Specimens from the Suez Canal collected by Hastings (1927), registered as *Amathia ?brasiliensis* (NHMUK 1926.9.6.25), have stems with similar diameters (0.18–0.20 mm) to those of *A. brasiliensis* Busk, but have smaller and broader free ends of zooids than the 'Challenger' specimens of *A. brasiliensis*. D'Hondt (1983) redescribed the Suez Canal material as *Amathia distans* var. *aegyptiana*, which was later raised to specific rank as *Amathia aegyptiana* by Chimonides (1987). The rooting processes are present in both *A. brasiliensis* and *A. aegyptiana* and the long bare terminal ramification, rarely present in *A. brasiliensis*, was not described for *A. aegyptiana*.

Chimonides (1987), although noticing some similarities among *Amathia pruvoti*, *A. distans*, *A. aegyptiana* and *A. brasiliensis*, observed several morphological characteristics that support each one as a distinct entity. These include the spiraling pattern of autozooidal groups (close to 360° in the last three species), the production of rhizoids only by *A. brasiliensis*, and the autozooidal spirality direction between maternal and daughter stolons. According to Chimonides (1987), *A. aegyptiana* is the only one that produces autozooidal groups in which the direction of the spirality is maintained. Another distinctive character of *A. aegyptiana* is the absence of rhizoids [according to Chimonides (1987) and Souto *et al.* (2010)], but this structure is inconstant among the different specimens of *A. brasiliensis* studied in the Western Atlantic and could represent ecophenotypical variation.

TABLE 2. Measurements (n = 10) and selected characters of *Amathia brasiliensis* Busk, 1886 from the Brazilian coast.

	NHMUK 1887.12.9.928	MZUSP 505	MZUSP 495	MZUSP 493	MZUSP 496	MZUSP 504
Autozooid length						
Min-Max	-	0.320–0.580	0.300–0.680	0.340–0.540	0.390–0.520	0.410–0.570
Mean (SD)	-	0.422 (0.068)	0.300 (0.124)	0.450 (0.058)	0.440 (0.046)	0.454 (0.050)
Autozooid width						
Min-Max	-	0.080–0.160	0.080–0.130	0.090–0.140	0.090–0.160	0.100–0.130
Mean (SD)	-	0.118 (0.024)	0.106 (0.16)	0.107 (0.020)	0.121 (0.019)	0.116 (0.008)
Stolon length						
Min-Max	-	1.131–1.755	1.404–2.145	1.365–2.028	1.482–2.145	1.872–2.340
Mean (SD)	-	1.446 (0.182)	1.720 (0.199)	1.692 (0.205)	1.841 (0.199)	2.086 (0.145)
Stolon width						
Min-Max	-	0.150–0.210	0.150–0.260	0.150–0.210	0.170–0.260	0.170–0.240
Mean (SD)	~ 0.22	0.179 (0.018)	0.199 (0.031)	0.177 (0.020)	0.216 (0.029)	0.196 (0.026)
Clusters of Autozooid length						
Min-Max	-	0.721–1.209	0.897–1.248	0.702–1.248	1.092–1.443	1.053–1.287
Mean (SD)	-	0.957 (0.162)	1.036 (0.101)	1.014 (0.115)	1.326 (0.112)	1.178 (0.075)
Proportion of clusters on stolon						
Min-Max	-	58%–79%	55%–67%	51%–74%	61%–82%	50%–64%
Mean (SD)	-	66% (7%)	60% (4%)	60% (8%)	72% (6%)	57% (5%)
Pairs of autozooids per cluster						
Min-Max	16–19	9–15	11–15	8–16	12–18	12–15
Spiral direction in daughter stolon						
Left stolon	as maternal stolon	as maternal stolon	clockwise	clockwise	clockwise	as maternal stolon
Right stolon	as maternal stolon	as maternal stolon	clockwise	clockwise	clockwise	as maternal stolon

TABLE 2. (continued.)

	MZUSP 494	MZUSP 492	MZUSP 507	MZUSP 497	MZUSP 506
Autozooid length					
Min-Max	0.400–0.540	0.320–0.530	0.340–0.480	0.290–0.470	0.380–0.480
Mean (SD)	0.469 (0.047)	0.432 (0.061)	0.412 (0.055)	0.382 (0.57)	0.446 (0.36)
Autozooid width					
Min-Max	0.090–0.150	0.110–0.150	0.110–0.150	0.090–0.130	0.100–0.130
Mean (SD)	0.118 (0.019)	0.124 (0.013)	0.127 (0.15)	0.110 (0.15)	0.112 (0.009)
Stolon length					
Min-Max	1.677–3.237	1.560–2.184	1.521–2.028	1.189–2.125	1.744–2.496
Mean (SD)	2.289 (0.480)	2.012 (0.196)	1.735 (0.165)	1.689 (0.236)	2.083 (0.293)
Stolon width					
Min-Max	0.170–0.220	0.170–0.250	0.140–0.230	0.160–0.230	0.210–0.260
Mean (SD)	0.199 (0.018)	0.205 (0.023)	0.187 (0.027)	0.191 (0.025)	0.230 (0.014)
Clusters of Autozooid length					
Min-Max	0.819–1.248	1.170–1.482	0.858–1.209	0.955–1.346	1.170–1.560
Mean (SD)	1.069 (0.137)	1.349 (0.105)	1.053 (0.113)	1.129 (0.104)	1.377 (0.140)
Proportion of clusters on stolon					
Min-Max	35%–70%	55%–80%	56%–66%	58%–80%	59%–80%
Mean (SD)	48% (10%)	67% (7%)	61% (3%)	68% (6%)	66% (6%)
Pairs of autozooids per cluster					
Min-Max	11–14	11–15	11–15	11–14	9–18
Spiral direction in daughter stolon					
Left stolon	as maternal stolon	Clockwise	as maternal stolon	anticlockwise	as maternal stolon
Right stolon	as maternal stolon	Clockwise	as maternal stolon	anticlockwise	as maternal stolon

Souto *et al.* (2010) described *Amathia minoricensis* from Balearic waters (Iberian Peninsula), in which the direction of twisting remains the same as that of the maternal stolons. Although *A. brasiliensis* and *A. minoricensis* present overlapping measurements for autozooids and stolon width [page 189 of Souto *et al.* (2010); Table 2 of this study], the latter species is readily distinguished by the helix of autozooids describing about 180–250°, the autozooids being joined for almost their total length, and the length of the stolon and zooid cluster being shorter in *A. brasiliensis* than in *A. minoricensis*. Souto *et al.* (2010) also noted differences between the spiraling direction of *A. aegyptiana* and *A. brasiliensis*, which according to them is not conserved along the colony of the latter species. However, in all specimens of *A. brasiliensis* here analyzed, including the type specimens, the direction of twisting is conserved along the colony. Busk (1886: p. 34) also noted for *A. brasiliensis* that the "comparative distinctness of the zooecia in the spiral series shows a tendency in the same direction".

Biological notes. In Brazil, *A. brasiliensis* is common on various substrata, including algae, bryozoans and anthropogenic surfaces, and was observed also on or with other vesiculariids, such as *A. distans*, *Amathia* cf. *vidovici*, *Amathia* cf. *crispa* and *Z. verticillatum*.

Distribution. Western Atlantic: North Carolina (Maturo 1957), Bermuda, Porto Rico (Osburn 1940), and Brazil (Vieira *et al.* 2008). Brazil: Fernando de Noronha Island, and states of Alagoas, Bahia, São Paulo and Santa Catarina.

Amathia distans Busk, 1886
(Figs 3, 4, 6, 8, 10; Tables 1 and 3)

Amathia distans Busk, 1886: 33, pl. 7, fig. 1; Marcus 1937: 134 (part) (not pl. 27, fig. 72); 1941: 28, fig. 29; 1949: 27, fig. 42; 1955: 313 (part); Osburn 1940: 339; Winston 1982: 110, fig. 10; d'Hondt 1983: p. 65 (part; form 1, p. 69).

Material examined. *Lectotype* (chosen here): NHMUK 1887.12.9.925 (Figures 3 and 4), *Amathia distans* (G. Busk det.), H.M.S. '*Challenger*', Bahia (Brazil), 10–20 fms (18.29–36.58 m), figured by Busk (1886, pl. vii, fig. 1). *Paralectotypes*: NHMUK 1887.12.9.926, *Amathia distans* (G. Busk det.), H.M.S. '*Challenger*', Bahia (Brazil), 10–20 fms (18.29–36.58 m); NHMUK 1899.7.1.4358–4359, *Amathia distans*, H.M.S. '*Challenger*', Bahia (Brazil), G. Busk Collection; NHMUK 1963.2.12.359, *Amathia distans*, H.M.S. '*Challenger*', Bahia (Brazil), Dundee Collection. Additional material (Table 1): MZUSP (498–500), MZUSP (508–510) (donated to NHMUK), São Sebastião, São Paulo State, Brazil.

Redescription. Colonies cuticularized, erect, regularly branched, transparent white to yellowish tan. Frontal surface of stolons and zooids of living colonies with bright yellow pigment spots. Autozooids in biserial clusters, 9 to 19 pairs of long clockwise and/or anticlockwise complete spirals at the distal end around the thickly cuticularized slender stolon; direction of spirals not maintained from maternal to daughter stolons; each maternal stolon buds two daughter stolons, one of which has autozooidal clusters organized in a clockwise spiral and the other in an anticlockwise spiral. Clusters present from distal half of stolon to three quarters of its length. Zooids tubular, connate for almost their entire length, not varying in size with the position of the series. Orifice terminal, circular. Polypides with 8 equal, slightly curved tentacles about 0.23 mm in length, with yellow pigment spots.

Remarks. *Amathia distans* is distinguished from its congeners by the combined presence of bright yellow pigment spots in stolonal and zooidal surfaces, including lophophores, and by a thickly cuticularized slender stolon (0.09–0.14 mm in diameter). The daughter stolons have autozooids organized in clockwise and anticlockwise directions, with the direction of spirals rarely maintained from maternal to daughter stolons as in *A. brasiliensis*.

Amathia distans and *A. brasiliensis*, which were described for the same locality by Busk (1886), were originally distinguished by the colony branching pattern, the stolon diameter and the size and position of the zooid clusters on the stolon. These distinctive characteristics have been confirmed through morphological analyses of syntype specimens deposited at the NHMUK. Here, we have selected and figured (Figs 1–4) the lectotype specimens of both *A. brasiliensis* (NHMUK 1887.12.9.928) and *A. distans* (NHMUK 1887.12.9.925).

Specimens attributed to *A. distans* by different researchers over the years show morphological characteristics that do not completely fit the original description of Busk (1886) and probably pertain to other species. Unfortunately, any potential pigmentation differences cannot be observed in preserved colonies, as the pigment is lost in alcohol; only the characters of zooids, zooid clusters and internodes are available for comparison. Harmer (1915) identified as *A. distans* specimens characterized by dichotomous colonies with zooids partially joined, usually forming one complete loop in the distal part of the slender internode, about 0.10 mm in diameter. Although the diameter of the stolons measured by Harmer (1915) is similar to that in *A. distans* Busk, the Siboga specimens have zooids unjoined for two-fifths or more of their length.

Several Brazilian colonies with both wider and slenderer stolons were identified by Marcus (1937, 1941, 1949, 1955) under the name *A. distans*. Figures based upon specimens collected in São Paulo State (Marcus 1937: pl. 27, fig. 72; Marcus 1949: fig. 42) show stolons 0.15–0.35 mm in diameter and were probably based upon both *A. distans* and *A. brasiliensis*. Osburn (1940) distinguished Caribbean specimens of *A. brasiliensis* and *A. distans* on the basis of stolon diameter of stolon and the degree of zooid joining. Yellow-pigmented zooids were first noted in *A. distans* collected in Florida by Winston (1982), and have never been reported in specimens of *A. distans* from the Pacific (D.P. Gordon, pers. comm.). The yellow-spotted Atlantic variety of *A. distans* (footnote 4, p. 69 in d'Hondt 1983) is considered *A. distans* sensu stricto. D'Hondt's figured specimen (d'Hondt 1983: p. 64, fig. 35C; p. 69, form 2) probably represents a different species, based on the presence of autozooids 0.20–0.25 mm in diameter, wider than those of *A. distans* Busk (Table 3). The small specimen from South Australia recorded as *A. distans* by MacGillivray (1895) has unjoined zooids; this specimen also probably belongs to a different species, which resembles '*Amathia distans* form 2' of d'Hondt (1983: p. 69).

Chimonides (1987) discussed other morphological similarities (e.g. shape of clusters of zooids rotating 360° around stolon) and differences (proportion of bare stolon between adjacent zooid clusters and diameter of stolon) between *A. brasiliensis* and *A. distans*. Souto *et al.* (2010) reported a similar appearance amongst *A. minoricensis* and some widespread species, including *A. distans*. However, according to those authors, *A. minoricensis* is readily distinguished from either *A. distans* or *A. brasiliensis* by the presence of rhizoids, autozooidal shape, degree of twisting of zooid clusters along stolons and the branching pattern of colonies.

Biological notes. *Amathia distans* sensu stricto occurs at least from Brazil to Florida on substrata including algae, bryozoans, and anthropogenic surfaces.

TABLE 3. Measurements (n = 10) and selected characters of *Amathia distans* Busk, 1886 from the Brazilian coast.

	NHMUK 1887.12.9.925	MZUSP 498	MZUSP 499	MZUSP 508	MZUSP 509	MZUSP 510
Autozooid length						
Min-Max	-	0.310–0.540	0.350–0.470	0.330–0.480	0.390–0.520	0.310–0.520
Mean (SD)	-	0.450 (0.070)	0.405 (0.032)	0.414 (0.041)	0.424 (0.039)	0.410 (0.060)
Autozooid width						
Min-Max	-	0.080–0.130	0.070–0.130	0.070–0.120	0.080–0.130	0.080–0.120
Mean (SD)	-	0.101 (0.014)	0.096 (0.018)	0.096 (0.016)	0.110 (0.015)	0.101 (0.012)
Stolon length						
Min-Max	-	1.715–2.340	1.833–2.379	2.028–2.934	1.872–2.652	1.833–2.691
Mean (SD)	-	2.114 (0.214)	2.118 (0.210)	2.410 (0.328)	2.301 (0.225)	2.324 (0.256)
Stolon width						
Min-Max	-	0.100–0.120	0.090–0.120	0.110–0.140	0.100–0.120	0.090–0.120
Mean (SD)	~ 0.12	0.111 (0.007)	0.103 (0.009)	0.119 (0.009)	0.112 (0.008)	0.107 (0.011)
Clusters of Autozooid length						
Min-Max	-	0.936–1.482	1.014–1.560	1.092–1.872	1.404–1.872	1.248–1.677
Mean (SD)	-	1.256 (0.153)	1.299 (0.179)	1.509 (0.217)	1.575 (0.153)	1.147 (0.170)
Proportion of clusters on stolon						
Min-Max	-	52%–73%	52%–68%	50%–74%	59%–80%	53%–74%
Mean (SD)	-	59 % (6%)	61% (4%)	62% (7%)	69% (7%)	64% (6%)
Pairs of autozooids per cluster						
Min-Max	12–14	11–16	11–16	12–23	9–16	10–19
Spiral direction in daughter stolon						
Left stolon	-	Clockwise	clockwise	clockwise anti-clockwise	clockwise anti-clockwise	clockwise
Right stolon	-	Anticlockwise	anticlockwise	anticlockwise clockwise	anticlockwise clockwise	anticlockwise

Distribution. The species was originally described from the Brazilian State of Bahia by Busk (1886: p. 33), and was reported for the shores of the States of Espírito Santo, Rio de Janeiro, São Paulo, Paraná (Marcus 1937, 1941, 1949, 1955), and Alagoas (Vieira *et al.* 2007, 2008). It has since been treated as a widespread fouling species in warm waters, with records for Australia, Indonesia, Florida, Southern California, Gulf of California, and New Zealand (viz. Harmer 1915; Gordon & Mawatari 1992; Soule *in* Osburn 1953; Menon 1972; Gordon, 2009; K. Tilbrook pers. comm. 2010.). However, some of these records are doubtful and require reinvestigation.

Results

DNA analyses. A 672 bp fragment of COI, ranging approximately from positions 9029–9701 of the *F. hispida* mitochondrial genome, was sequenced for ten colonies of *Amathia brasiliensis*, five of *A. distans*, two of *Amathia* cf. *crispa*, and four of *Amathia* cf. *vidovici* (Table 1). The sequences were translated with the invertebrate mitochondrial genetic code available in Geneious, and no stop or ambiguous codons were observed. Two haplotypes were found for *A. brasiliensis*: 'haplotype 1' from Maceió, São Sebastião and Ilhabela, and 'haplotype 2' from São Francisco do Sul and São Sebastião (Pi ~ 0.009; number of polymorphic sites = 6; one transition at 1st, one at 2nd, and four at 3rd positions). The comparisons among the aligned sequences of *A. brasiliensis* and *A. distans* resulted in 592 (~88.10%) invariable and 80 (~11.90%) variable sites (Table 4). Of the 224 codons analyzed, 67 synonymous and 14 replacement changes were observed.

TABLE 4. Sequence divergence between species for COI and 16S rRNA, expressed as percentage values of variable sites.

	Amathia cf. *crispa*	A. distans	*Amathia* cf. *vidovici*
A. brasiliensis	COI: 16.70	COI: 11.90	COI: 11.00
	16S: 14.00	16S: 11.80	16S: 08.00
Amathia cf. *crispa*		COI: 16.10	COI: 15.90
		16S: 16.70	16S: 11.50
A. distans			COI: 09.80
			16S: 12.30

A 522–537bp fragment of 16S rRNA, starting approximately at position 4129 of the *F. hispida* mitochondrial genome, was sequenced for eight colonies of *A. brasiliensis*, four of *A. distans*, two of *Amathia* cf. *crispa*, and four of *Amathia* cf. *vidovici* (Table 1). The pairwise analyses of 517 bp of *A. brasiliensis* and *A. distans* (after the exclusion of sites with gaps/missing data) computed 456 (~88.20%) invariable and 61 (~11.80%) variable sites (Table 4). Interestingly, a very similar proportion of invariable/variable sites was found for the two segments of genes analyzed. Considering COI and 16S rRNA, each species is characterized by unshared haplotypes with deep genetic divergence among them (Table 4). Hereby, the partitions for BI phylogenetic search included five haplotypes of COI and four of 16S rRNA as ingroup terminals.

The results of the combined BI analysis of COI + 16S rRNA support the monophyly of *A. brasiliensis* as well as the genealogical species status of that species and that of *A. distans* (Figure 11). In this tree, *A. distans* is the sister-group of (*Amathia* cf. *crispa* + (*A. brasiliensis* + *Amathia* cf. *vidovici*)). The placement of *A. distans* is supported (pp = 1.00). On the other hand, the phylogenetic position of *Amathia* cf. *crispa* (pp = 0.85) and *A. brasiliensis* + *Amathia* cf. *vidovici* (pp = 0.92) are not significantly supported, and hence should be considered as uncertain. Morphologically, *A. brasiliensis* + *Amathia* cf. *vidovici* share the presence of autozooid clusters that are unjoined along almost their entire length.
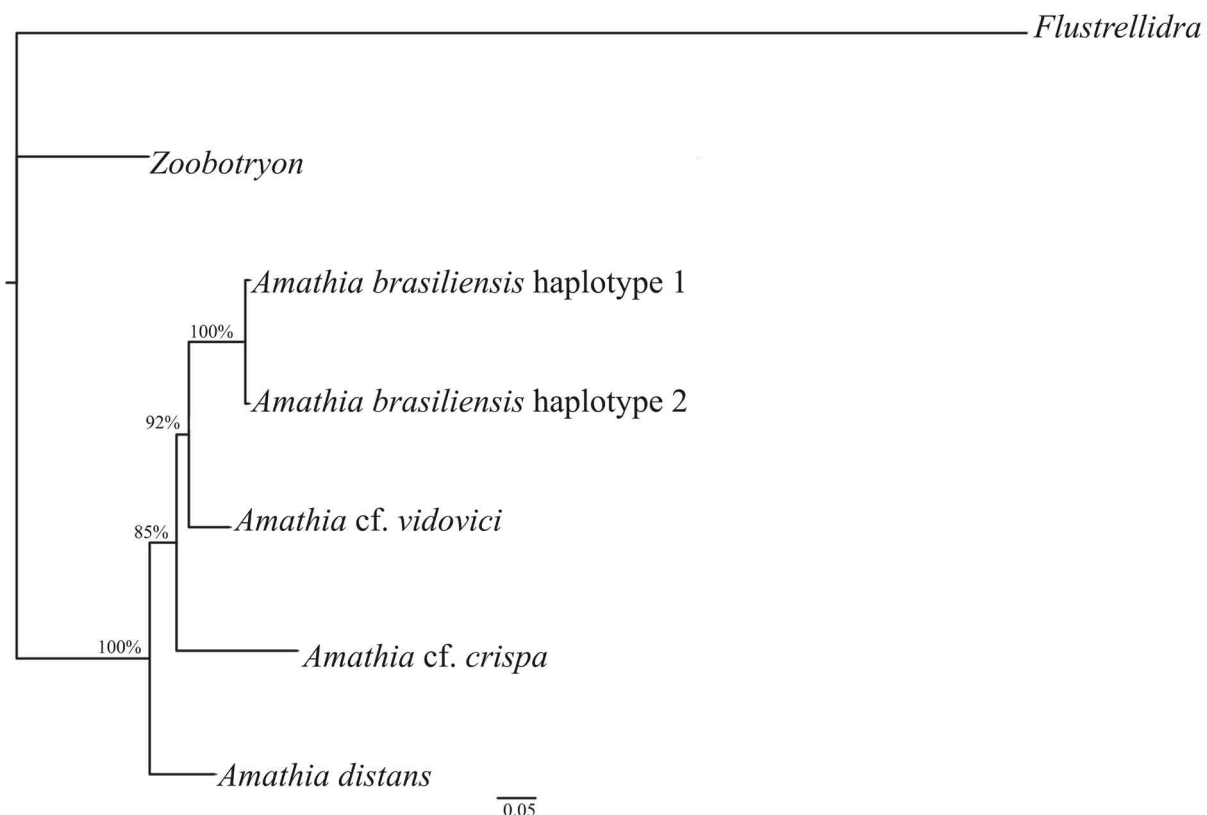
FIGURE 11. Bayesian phylogenetic tree of COI + 16S rRNA. Posterior probability values (pp < 95% judged a not significant) are depicted in nodes.

Discussion

At low taxonomic levels, the practice of adopting DNA sequences as a source of information has proven to be useful in identifying species lineages of bryozoans, especially for those groups for which analyses of morphological characters alone seem to be unsatisfactory. The existence of cryptic species complexes for *Membranipora membranacea* (Schwaninger 1999, 2008), *Celleporella hyalina* (Hoare *et al.* 2001), *Electra* (Nikulina *et al.* 2007; Nikulina 2008) and *Bugula neritina* (Davidson & Haygood 1999; McGovern & Hellberg 2003) was confirmed only after performing phylogenetic analyses of DNA sequence data, including segments of the genes COI, 16S rRNA and/or 18S rRNA. Finally, in a taxonomic study of *Alcyonidium*, Porter (2004) used DNA sequence comparisons of COI and 12S rRNA in conjunction with morphological and ecological information in the description of new species.

Similarly, the molecular analyses used here in the redescription of *A. brasiliensis* and *A. distans* also proved to be complementary to morphological data. Although it was not possible to access specimens of *A. brasiliensis* and *A. distans* from their type locality (the Brazilian State of Bahia), the selection of lectotypes, morphological observations, and analyses of DNA sequences contributed to the refinement of the taxonomy of both species. The morphological characters, permitting the identification of living and/or preserved specimens, are as follows: (1) *A. brasiliensis*: whitish-pale pigment spots in the frontal surface of stolons and zooids, and a wide stolon with biserial zooid clusters growing in clockwise and anti-clockwise spirals along it, with the direction of spirality maintained from maternal to daughter stolons; (2) *A. distans*: bright yellow pigment spots in stolonal and zooidal surfaces including lophophores, and a thickly cuticularized slender stolon with biserial zooid clusters in clockwise and anticlockwise spirals along it, with the direction of spirality not maintained from maternal to daughter stolons. Additionally, pairwise DNA sequence comparisons of COI and 16S rRNA revealed deep genetic divergence between the two species (~11% for both genes), and the BI tree supported their genealogical species status.

The low number of terminals and the non-significant support achieved for most nodes in the BI tree discouraged the approach of character mapping of morphological features onto the gene tree for this study; however, it is recommended that this practice be adopted in future studies regarding more inclusive questions on the interrelationships of *Amathia* in order to test the validity of the characters traditionally used in systematics. Finally, the expectation is that this work might serve as a starting point for future studies addressing the taxonomy, phylogeny and phylogeography of *Amathia* species and other vesiculariid taxa such as the outgroup *Zoobotryon verticillatum*, which is currently regarded as a single, cosmopolitan and bioinvasive species (e.g. Farrapeira 2011).

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